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High-speed high-performance liquid chromatography of peptides and proteins

H. Chen, Cs. Horváth*

Department of Chemical Engineering, Yale University, New Haven, CT 06520, USA

Abstract

Over the last thirty years the name HPLC has been synonymous with high-speed liquid chromatography and during the last ten years we have experienced a dramatic increase in the speed of analysis particularly as far as the separation of biological macromolecules, such as proteins, is concerned. With a solid grounding in the chromatographic theories, column technology has been mainly responsible for the advances in this field. Recent development shows that columns packed with micropellicular or gigaporous stationary phases of the bidisperse or the bimodal type facilitate rapid mass transfer between the mobile and stationary phases and thus can deliver high resolution separations in a very short time. This suggests that HPLC has the potential to be the prime analytical technique for on-line monitoring of biotechnological processes in real time. Further enhancement of the speed of separation comes from the use of elevated temperatures. The role of temperature in HPLC has largely been ignored and most commercial instruments are not equipped with appropriate temperature control. Results presented here strongly suggest, however, that elevated column temperature may find increasing use in the HPLC of large molecules. In such analytical applications temperature programming may also play a major role provided columns with low heat capacity, such as packed fused-silica capillaries, gain wider employment in HPLC.

Contents

* Corresponding author.

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1. Introduction

The development of the technique, which we call by the acronym HPLC, commenced about 30 years ago [1]. At that time the high pressure was the dominant new operational and instrumental characteristic and the relatively high speed of the technique was the most important measure of performance. For this reason the name "high-speed liquid chromatography" was also used concurrently with "high-performance liquid chromatography" to denote the new technique [2,3]. Although it has acquired over the years all features associated with high performance, the high speed of separation is still a cardinal feature of HPLC.

High-speed HPLC has been widely used in routine analytical work, method development, process monitoring and quality control, and further progress in these area depends on the development of efficient means for more rapid separations. It is particularly important due to the sequential nature of the separation by column chromatography that allows the separation of only one sample at the time in contrast to planar chromatographic and electrophoretic techniques which facilitates simultaneous separation of several samples. With advances in data acquisition and processing, sample preparation and the chromatographic separation steps are the weakest links in the chain of analytical information gathering and transfer as shown in Fig. 1. Since the preparation of a large number of samples can be carried out parallel, however, the ultimate limiting step remains the chromatographic separation per se.

Recently, process monitoring and control have drawn attention in biotechnology in response to the requirements by federal regulatory agencies [4]. At the present, mostly off-line analytical procedures [5], which may take several hours or even days, are employed for "in-process analysis". The need for on-line monitoring by rapid analytical methods is increasingly recognized to carry out real time monitoring and thus avoid the loss of product and to reduce the total process time and cost [6–8].

Recent advances in HPLC with concomitant

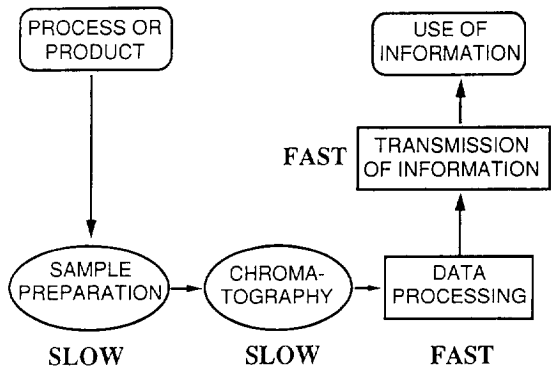

Fig. 1. Schematic illustration of the sample and information flow. Due to the sequential nature of sample handling by column chromatography, HPLC separation is usually the rate-limiting step of the processes. Sample preparation can be carried out in a parallel fashion so that a large number of samples can be simultaneously processed.

enhancement of the separation speed and efficiency strongly suggest that HPLC has the potential to become an important method for on-line monitoring [9]. Although much is expected from the development of various sensors [10] for such purposes, the capability for rapid sequential multicomponent analysis makes a suitable liquid chromatograph to be the ultimate sensor, particularly when the advances anticipated in miniaturization lead to a new generation of instruments. As a separation technique with high resolving power, HPLC is superior in monitoring the concentration of several components over other conventional on-line methods, such as spectroscopic or electrochemical measurements. In the past few years, different approaches have already been developed to monitor by HPLC the fermentation process [11–14] or the effluents from down-stream processing operations [15–17]. By and large HPLC is poised to become a quasi-real time monitoring tool for processes in biotechnology.

Rapid analytical HPLC methods will offer significant advantages also in routine analytical work in the laboratory. Furthermore, analytical HPLC is increasingly used in molecular chromatography to obtain information on the structure of biopolymers, such as proteins, and to study

molecular interactions as well as the chromatographic process proper at the molecular level [18–20]. By increasing the speed of analysis, the analytical productivity and efficiency of both the operator and the instrument can greatly be improved. On the other hand, multidimensional liquid chromatography with coupled columns [21] entails a large number of individual chromatographic runs and is therefore, greatly limited today by the total amount of time required for a complete analysis. Further reduction in the time required for each of the separation steps is necessary to develop the full potential of this highly promising technique and to make multidimensional HPLC, with appropriate automated instrumentation, a powerful analytical method that can be widely used for the determination of the composition of samples containing many components.

In the present report, we shall discuss recent advances and some approaches to achieve rapid analysis of peptides and proteins by HPLC, the peculiarities of the attendant instrumentation and the operational parameters of high-speed HPLC.

2. Theory

The time of chromatographic analysis can be defined as the retention time, t_R, of the most retained sample component. Under isocratic conditions it is given by

$$t_R = \frac{L}{u} \cdot (1 + k') \tag{1}$$

where k' is the retention factor of the last peak, L is the column length and u is the linear flow velocity of the mobile phase. The column length depends on the number of theoretical plates, N, required for the multicomponent separation and on the reduced plate height, h, as

$$L = Nd_p h \tag{2}$$

where d_p is the particle diameter. On the other hand u can be expressed by the reduced flow velocity, ν, which is also termed Peclet number in the chemical engineering literature, as

$$u = \frac{D_m \nu}{d_p} \tag{3}$$

where D_m is the eluite diffusivity in the mobile phase.

According to Darcy's law, the pressure drop across the column is

$$\Delta P = \frac{uL\eta\psi}{d_p^2} \tag{4}$$

where ΔP is the pressure drop and η is the viscosity of mobile phase. The parameter ψ is given by $180(1+\omega)(1-\epsilon)^2/\epsilon^2$, where ϵ is the interstitial porosity, 0.4 for random packing, and ω is the volume ratio of the intraparticulate and interstitial void spaces. The latter can be assumed to be unity for totally porous and negligibly small for pellicular sorbents.

For fixed pressure drop and plate number, the shortest analysis time, t_R^*, is achieved at the optimum flow velocity, ν_0 [22] at which the reduced plate height has its minimum value, h_0. Under such conditions t_R^* can be expressed as

$$t_R^* = \frac{N^2 h_0^2 \eta\psi}{\Delta P} \cdot (1 + k') \tag{5}$$

and the particle diameter d_p is obtained as

$$d_p = \sqrt{\frac{h_0 \nu_0 N D_m \eta\psi}{\Delta P}} \tag{6}$$

In the chromatography of small molecules, D_m can be taken as $1 \cdot 10^{-5}$ cm^2/s. If we set N to 5000, ΔP to 300 bar, k' to 3, h_0 to 2, ν_0 to 3 and η to 10^{-2} g cm^{-1} s^{-1}, the separation time calculated from Eq. 5 is approximately 10 s. In this particular case the column length, flow velocity and particle size were evaluated as 1 cm, 0.3 cm/s and 1 μm, respectively, by using Eqs. 2, 3 and 6 with the minimum reduced plate height and optimum reduced velocity.

In the chromatography of large molecules having much smaller diffusivities, however, the requirements may change dramatically. If we use D_m of $5 \cdot 10^{-7}$ cm^2/s instead of $1 \cdot 10^{-5}$ cm^2/s in the case above, we find that for an analysis time

of 10 s, the column length, flow velocity and particle size typically required in macromolecular chromatography would be 0.2 cm, 0.08 cm/s and 0.2 μm, respectively. Whereas such particles are too small for use in packed columns at present, a thick membrane or a stack of thin membranes having 0.2-μm pores, suitable retentive properties, and favorable pore morphology could serve as a chromatographic column for such rapid separations [23].

In practice, the separation of large molecules is generally carried out by using gradient elution. Under linear solvent strength gradient conditions, the analysis time can be estimated by the relationship [24]

$$t_R \approx \frac{L}{u} + \frac{t_G}{1 - \frac{k_f}{k_0}} \tag{7}$$

where t_G is the gradient time and k_0 and k_f are the respective isocratic retention factors of the most retained sample component at the initial and final mobile phase compositions.

Under gradient conditions the plate number of the column can be estimated at high flow velocities by using the expression [25]

$$N \approx \frac{t_G D_m}{1.15 C' d_p^2 \bar{k} \log \frac{k_0}{k_f}} \tag{8}$$

where $\bar{k}$ is the retention factor at the midpoint of the column and the parameter C' depends on the porosity and structure of the packing, the equilibrium constant and the stationary phase configuration, which represents the particular form and structure of the support and the stationary phase proper. Certain novel stationary phase configurations, such as pellicular, gigaporous, and gel-in-a-shell type are of interest in high-speed HPLC of large molecules and will be discussed later.

At fixed plate number and the pressure drop, the advantages of small particle size for a given separation are evident from Eqs. 4 and 8. Both the column length and gradient time decrease with d_p^2 and as a result the reduction in d_p yields fast separations as seen from Eq. 7. However, there is a lower practical limit for the particle diameter and therefore further gains would require the use of stationary phases of special configuration that yields small values of parameter C' in Eq. 8. The resolution in gradient elution does not depend on the column length and the flow velocity [25], thus, flow velocity can be increased, and at the same time the column length reduced, to achieve fast separations at a fixed column inlet pressure.

As seen from Eq. 4, the mobile phase velocity, at the fixed pressure drop, can be increased upon reducing the eluent viscosity by using elevated temperature and thus practicing "superheated" liquid chromatography [26] or even supercritical fluid chromatography with eluents commonly employed in HPLC. Such conditions also lead to an enhancement of the eluite diffusivity in the mobile phase, thus to a reduction of the time of analysis with the same column at a fixed plate number according to Eq. 8 provided the stationary phase is stable at elevated temperatures.

In summary, the employment of small particles, stationary phase configurations with favorable mass transfer properties, and elevated column temperatures combined with the use of steep gradients, high flow velocities, and short columns can be a very effective means to bring about rapid separations of large molecules, such as peptides and proteins.

3. Column design

3.1. Particle size

The effect of the particle size on the analysis time is well understood when using columns packed with conventional sorbents [22,27–30]. Small particles yield enhanced column efficiency by virtue of the relatively small intraparticulate mass transfer resistance due to the short diffusion distances and to a lesser extent due to the small contribution of "eddy diffusion" to the plate height [31]. The enhancement of intraparticular mass transport is particularly important for the rapid separation of large molecules having low diffusivities.

For fixed plate number the retention time can be calculated as a function of particle diameter d_p by combining Eqs. 1–3 as

$$t_R = \frac{(1+k')Nh}{D_m \nu} \cdot d_p^2 \tag{9}$$

As we mentioned in the previous section, high-speed chromatography of large molecules is often carried out at high reduced velocities. Under such conditions the magnitude of intraparticle diffusion resistances in columns packed with porous adsorbents determines the dependence of the reduced plate height on the reduced velocity. In certain practical cases h can be considered to be linearly dependent on ν and the slope of the h vs. ν plot at $k' = 3$ is found to be approximately 0.3 [32]. The results obtained by using Eq. 9 with this assumption and for a given set of practical conditions used in macromolecular separations are shown in Fig. 2. It is seen that columns packed with 1–3-μm monodisperse particles can yield 2000 theoretical plates within a few minutes in the separation of large molecules.

Columns packed with 1.5-μm [33] and 2-μm [34] pellicular as well as 2-μm [35] porous stationary phase particles have been employed in rapid analysis of peptides and proteins. Columns packed with small particles, however, have low permeability and require high column inlet pressure at flow velocities employed for fast separations. The inlet pressure required for columns packed with conventional porous spherical stationary phase particles and operated at high reduced velocities can be determined by

$$\Delta P = \frac{L^2 \eta D_m \psi}{0.3 N d_p^4} \tag{10}$$

The dependence of the column inlet pressure on the particle diameter was calculated by Eq. 10 and the results are also depicted in Fig. 2. Since the pressure is inversely proportional to the fourth power of particle diameter, it increases rapidly with decreasing particle size. As a result the potential of microparticulate stationary phases in HPLC of large molecules is limited by the low column permeability. In such cases, it may be particularly appropriate to reduce the viscosity of the mobile phase by carrying out chromatography at elevated column temperatures with columns having adequate stability for operation at high temperatures. The peculiarities of HPLC at high temperatures will be discussed later in this paper.

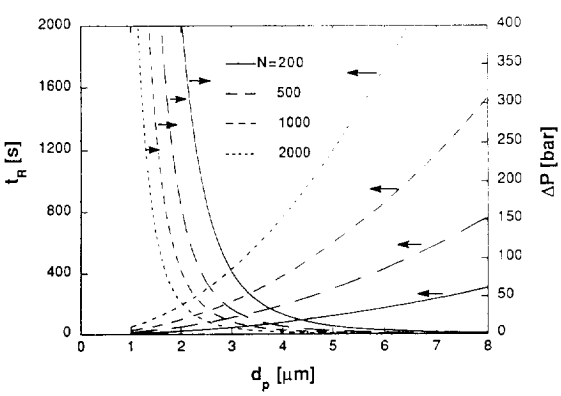

Fig. 2. Dependence of analysis time and pressure drop on particle size at a given plate number for totally porous materials. $k' = 3$, $h = 0.3\nu$, $D_m = 5 \cdot 10^{-7}$ cm^2/s, $L = 3$ cm, $\eta = 0.9$ cP, $\epsilon = 0.4$, and the required N is shown in the figure.

3.2. Stationary phases of novel configuration

In addition to the particle size, the configuration of the stationary phase also plays an important role in determining the speed and efficiency of separation. For rapid HPLC stationary phases of novel configuration, e.g., micropellicular, gigaporous, or gel-in-a-shell type were developed during the past few years and found to have mass transfer properties superior to conventional column packings. Indeed, columns packed with such stationary phases are eminently suitable for the HPLC of biological macromolecules at high efficiency and high speed.

3.2.1. Micropellicular stationary phases

Pellicular stationary phases with an average particle size of 40 μm were first introduced in the mid-1960s [36] after the felicitous experience gained with such stationary phase structure in gas chromatography [37]. Whereas pellicular

stationary phases dominated HPLC in the early years, they were largely replaced in the seventies by totally porous microparticulate bonded phases for the separations of small molecules upon the availability of high performance air classifiers for subsieve particles. Micropellicular stationary phases having small particle diameter (d_p = 1.5–5 μm) shown in Fig. 3 regained interest in the middle eighties for the rapid HPLC of biopolymers [33,34,38–40] and are expected to play an important role in analytical work.

As shown in Fig. 3 the configuration of the actual stationary phase in micropellicular packings is a spherical annulus supported by a fluid impervious, rigid microsphere. The main advantage of such sorbents rests with the rapid mass transfer between the mobile and stationary phases because the diffusion distance in the thin retentive layer of the particles is short. Owing to the solid, fluid-impervious core of the micropellicular packings, columns are stable at high pressures and elevated temperatures unlike those packed with conventional porous materials. Moreover, they also provide improved recoveries in the separation of proteins and peptides. The cavernous interior of conventional sorbents is considered to be responsible for the entrapment of sample. The loading capacity of columns packed with micropellicular stationary phases is relatively low with small molecules, yet with macromolecules it is not much smaller than the loading capacity of totally porous sorbents

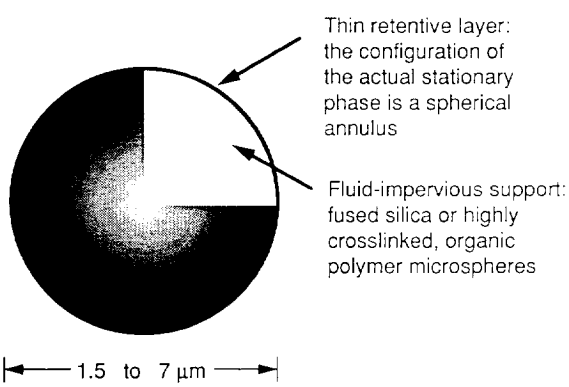

Fig. 3. A schematic illustration of the pellicular stationary phase configuration.

[41]. Because of the small particle diameter (1.5–3 μm) of the micropellicular packings, the permeability of the column is low. This impediment, however, can be mitigated by carrying out the separation at elevated temperature at which the mobile phase viscosity is reduced. The combination of elevated column temperature and the micropellicular stationary phase configuration offers a powerful means to facilitate rapid and efficient separation of large molecules [34,42]. Fast separation of a mixture of four proteins depicted in Fig. 4 was achieved in 6 s at 120°C on a 3-cm HY-TACH column packed with 2-μm pellicular ODS-silica. Fig. 5 shows peptide profiles of tryptic digests of β-lactoglobulin A and methionyl human growth hormone produced by recombinant DNA technology. The chromatograms were obtained on a HY-TACH column similar to that used in Fig. 4. Whereas the quality of the separation does not meet the high standards of the tryptic maps used in protein analy-

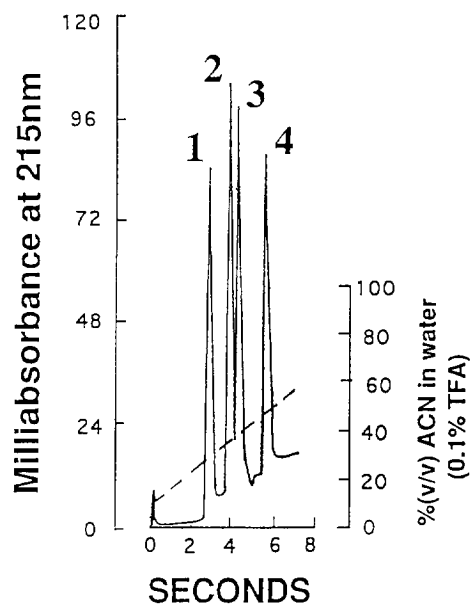

Fig. 4. Rapid separation of standard proteins. Column, 30 × 4.6 mm, packed with 2-μm pellicular ODS-silica; 12 s linear gradient from 10 to 90% (v/v) acetonitrile (ACN) in water containing 0.1% (v/v) trifluoroacetic acid (TFA); temperature, 120°C; flow-rate, 5 ml/min; column inlet pressure, 240 bar. Peaks: 1 = ribonuclease A; 2 = cytochrome *c*; 3 = lysozyme; 4 = β-lactoglobulin B. From Ref. [42].

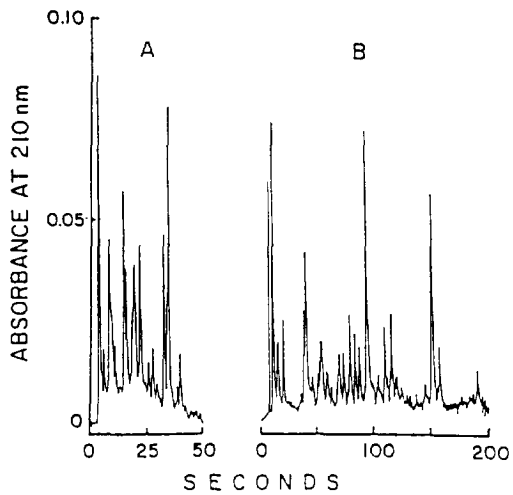

Fig. 5. Chromatographic profiles of tryptic digests of (A) β-lactoglobulin A and (B) methionyl human growth hormone. Column, 30 × 4.6 mm, packed with 2-μm pellicular ODS-silica; linear gradient from 0 to 95% (v/v) acetonitrile in water containing 0.1% (v/v) TFA in (A) 2 min at 5 ml/min and (B) 6 min at 4 ml/min; temperature, 80°C; samples, 5 μg of protein digest each. From Ref. [39].

sis, the time of separation is only a very small fraction of that required by conventional means.

3.2.2. Gigaporous packings

More recently, supports having pore diameters greater than one hundredth of the particle diameter were introduced for fast protein chromatography [43–45]. So far two major types of such gigaporous stationary phases have received great attention. One is prepared by using a bidisperse rigid gigaporous support with an appropriate retentive surface and the column is operated under conditions when mass transfer inside the stationary phase particles involves both intraparticle convection in the gigapores and diffusive transport in the smaller pores. The convective transport in the gigapores is often referred to in the literature as perfusion and such particles are termed perfusive [43]. The other type has a so called "gel-in-a-shell" configuration which is described as a rigid gigaporous support particle filled with a retentive hydrogel [46]. Unlike conventional packed columns, the efficiency of columns packed with the first type of stationary phase does not deteriorate much at high flow velocities even with proteins under conditions of no adsorption. This feature which greatly facilitates high-speed separation of biological macromolecules is attributed to intraparticle convection in the gigapores [43,47]. A schematic illustration of the cross-section of such stationary phase particles is presented in Fig. 6. Columns with gigaporous packings of this type usually have significantly lower loading capacities despite the presence of the small pore regions than those packed with conventional porous column materials.

The favorable mass transfer properties of gigaporous supports manifest in reduced intraparticle mass transfer resistances so that the corresponding reduced plate height increment, h_{intra}, is given by [46]

$$h_{intra} = C \cdot \frac{D'_e}{D_{app}} \cdot \nu \tag{11}$$

As discussed earlier, the magnitude of h_{intra} dominates the reduced plate height at high flow velocities in columns packed with conventional porous materials and the value of h_{intra} is negligible in columns packed with pellicular sorbents. The effects of intraparticle convection is measured appropriately by the relation between the effective diffusivity in the gigapores, D'_e, and the apparent effective diffusivity, D_{app}, which incorporates the effects of both diffusion and convection in the gigapores of a spherical par-

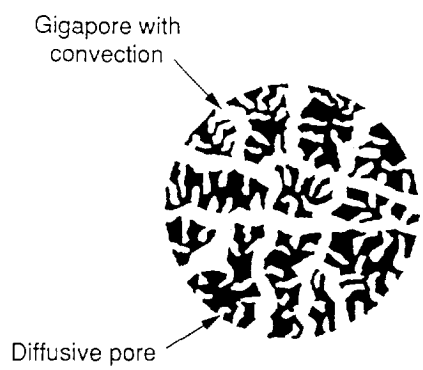

Fig. 6. A schematic illustration of the bidisperse gigaporous stationary phase configuration. After Ref. [43].

ticle [43,47,48]. The latest of these theoretical relationships [47] is given by

$$D_{app} = f\left(1 + \frac{2\nu'}{45}\right)D'_e \qquad (12)$$

where ν' is the reduced velocity in the gigapores and f is the correction factor for diffusive transport in the small pores.

The advantages of gigaporous bidisperse stationary phases are most evident at high flow velocities where the favorable effect of intraparticle convection on the apparent diffusivity is greater than at low flow velocities. The effect depends on the ratio of the diameter of the gigapores to the particle diameter that is assumed to be proportional to the square root of the ratio of the intraparticle flow velocity to the interstitial flow velocity. In Fig. 7 the theoretical dependence of h on ν is illustrated in the absence of intraparticle convection and for three cases with different gigapore to particle diameter ratios as well as for pellicular packings with a chromatographic surface completely exposed to the eluent stream perfusing the column.

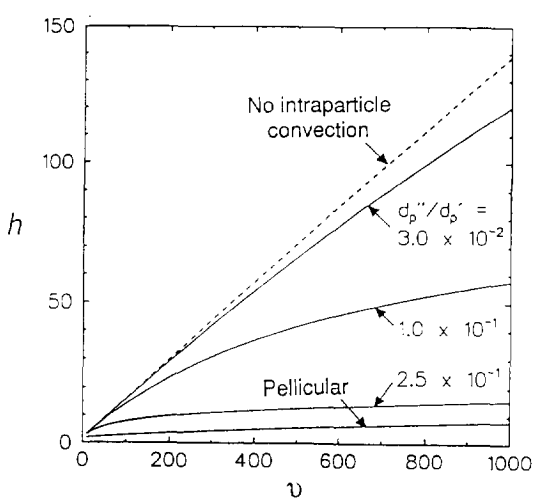

Fig. 7. Illustration of the reduced plate height for a retained eluite ($k' = 2$) as a function of the reduced velocity for various types of column packings. For gigaporous particles, the increasing level of intraparticle convection is expressed by increasing values of the ratio d_p''/d_p', which is equivalent to the ratio of the mean gigapore diameter to the mean interstitial channel diameter of the column packing. From Ref. [47].

The reduced mass transfer resistance in open gigaporous packings allows the use of relatively large particles and high flow velocities thus facilitates rapid analysis of large molecules. This is illustrated in Fig. 8 by the fast separation of a standard protein mixture on a 3 cm long column packed with 20-μm gigaporous adsorbents [16]. It has been shown that the capacity of the column packed with such gigaporous material of bidisperse pore structure does not change strongly with the flow velocity [43], and as a result,

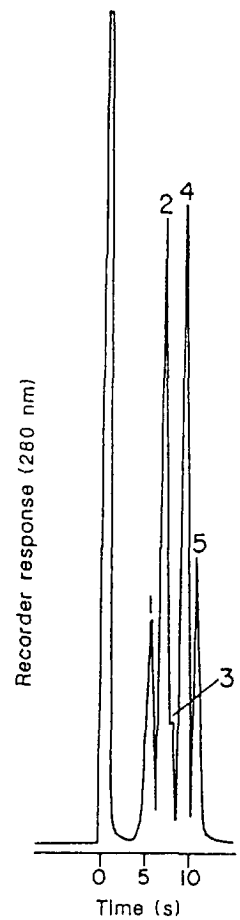

Fig. 8. Reversed-phase separation of proteins. Column, 30 × 2.1 mm, packed with 20-μm highly cross-linked styrene–divinylbenzene having 6000 to 8000 Å gigapores; gradient time, 24 s; flow-rate, 5 ml/min. Peaks: 1 = ribonuclease A; 2 = cytochrome c; 3 = lysozyme; 4 = β-lactoglobulin; 5 = ovabumin. From Ref. [16].

columns packed with such material find use in high-speed separation of peptides and proteins in both analytical and preparative/process-scale applications with modest plate requirements. Nevertheless, in the latter application, the loading capacity of columns packed with open gigaporous stationary phases may be still considered low despite their enhanced mass transfer properties due to intraparticulate convection.

The potential of gigaporous sorbents for rapid chromatography has not yet been fully exploited. For instance, gigaporous silica particles have been available for some time (cf. Table 1), yet, there is a paucity of literature data on their use for fast separation of substances having high molecular mass. Similarly, commercially available gigaporous ceramic hydroxyapatite may also be a useful stationary phase for rapid separation of proteins and nucleic acids.

A recent approach addresses the problem of low capacity by filling the open pores in the rigid support particles with a hydrogel which contains appropriate retentive sites and is permeable at least in part to the biopolymers to be separated. This novel stationary phase configuration shown in Fig. 9 is claimed to combine high loading capacity of the soft gels with the mechanical stability of the rigid particles so that the column can be operated at relatively high flow velocities [46]. Columns packed with this type of stationary phase may offer a felicitous combination of loading capacity, efficiency, speed, resolution, recovery and stability in comparison to the conventional stationary phases [45].

The novel stationary phase configurations of enhanced mass transfer properties described above are expected to find most efficient use in different types of applications where their potential can be fully exploited. Columns packed with pellicular sorbents are the best in obtaining rapid and high resolution analytical separations of biomacromolecules. Their relatively low loading capacity, however, impedes their employment in preparative separations with the exception of micropreparative applications where high speed and efficiency are required [49]. Columns packed with bidisperse gigaporous particles are designed for separations faster than those obtainable with conventional porous materials. They are particularly suited for rapid separations which do not require high efficiency as shown in Fig. 10 [47]. The advantages of such columns are manifest in high-speed analytical separations as well as in preparative-scale purifications. Columns packed with gel-in-a-shell stationary phases may have significantly higher loading capacity than those packed with open bidisperse gigaporous sorbents but their efficiency likely to diminish much faster upon increasing the flow velocity beyond a presently practical value. Thus both types of gigaporous sorbents are expected to find applications in high-speed, large-scale purification of proteins.

3.3. Column dimensions

Several studies have suggested that the resolution of macromolecules is rather insensitive to column length and flow velocity under conditions of gradient elution [25,28,50,51]. Therefore fast separation of peptides and proteins is usually carried out by gradient elution with short columns at high flow velocities often at the maximum permissible column inlet pressure of the system as discussed in the Theory section. Since the number of theoretical plates increases only with the square root of column length under isocratic conditions, also in this case the length of the column should be kept at a minimum for fast separations. The separation of β-lactoglobulin A and B within 15 s on a 1 cm long HYTACH column packed with micropellicular octadecyl-silica under isocratic elution conditions is illustrated in Fig. 11 [34]. This seems to be an extreme case of short columns because in general, 3–5 cm long columns are used for the separations of proteins in contrast with the 10–25 cm long columns employed for analysis of peptide mixtures [51].

The column diameter in the present practice of HPLC ranges mostly from 3 to 5 mm. However, the use of narrow-bore columns having a diameter between 0.5 and 2 mm and micro-bore columns with a diameter smaller than 0.5 mm is gaining significance. Columns with 1 mm inner diameter were used already in the mid-1960s [36]

Table 1
Some commercially available gigaporous silica supports

Trade name	Manufacturer	Mean pore diameter (Å)	Specific surface area (m^2/g)	Specific pore volume (ml/g)	Particle diameter (μm)	Maximum column pressure (p.s.i.)
Zorbax PSM-1000	Rockland Technologies, DE, USA	1000	15	0.75	5	4000
Nucleosil 1000	Macherey–Nagel, Düren, Germany	1000	25	0.8	5, 7, 10	3000
Nucleosil 4000	Macherey–Nagel	4000	10	0.7	5, 7, 10	3000
LiChrospher 1000	Merck, Darmstadt, Germany	1000	30	0.78	10	3000
LiChrospher 4000	Merck	4000	10	0.78	10	3000
SynChropak 1000	SynChrom, IN, USA	1000	25	0.8	7	3000
SynChropak 4000	SynChrom	4000	10	0.7	10	3000

1 p.s.i. = 6894.76 Pa.

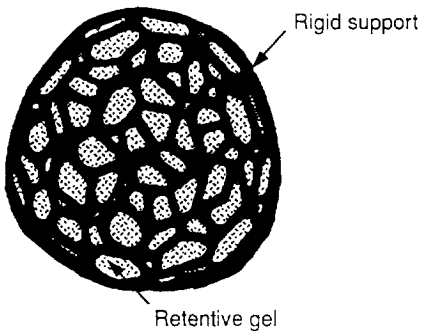

Fig. 9. Artist's rendition of the so called "gel-in-a-shell" stationary phase configuration.

but the instrumental constraints hampered the general acceptance of such narrow-bore columns at the dawn of HPLC. The broad impact of fused-silica capillaries on the practice of gas chromatography and capillary electrophoresis is likely to make capillary liquid chromatography, i.e., micro HPLC with packed quartz capillaries, the method of choice for analytical HPLC. The miniaturization of the column diameter will per se probably not augment separation speed or

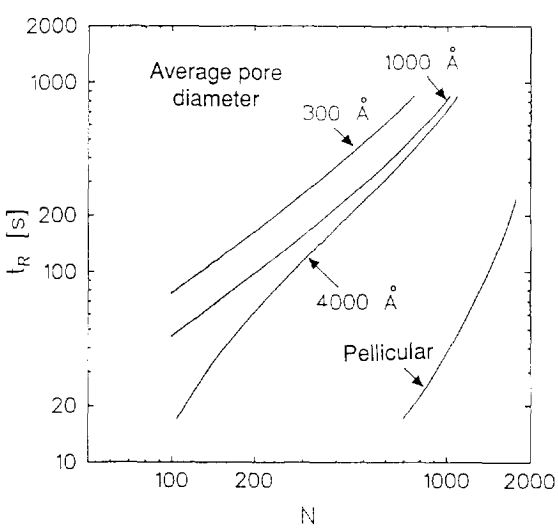

Fig. 10. Separation time versus plate number calculated for 5 cm long columns packed with 8-μm particles of various pore sizes as indicated. Conditions correspond to $D_m = 10^{-6}$ cm^2/s, molecular diameter of the eluite is 40 Å, $k' = 3.3$, and the ratio of intraparticulate and interstitial flow velocities $u'/u = 10^{-2}$. From Ref. [47].

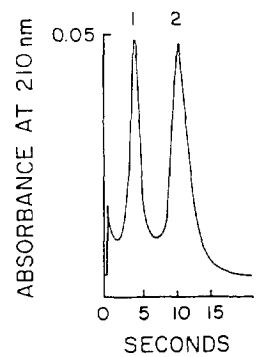

Fig. 11. Separation of β-lactoglobulin B and A by isocratic elution. Column, 10 × 4.6 mm, packed with the micropellicular sorbent; eluent, 36.75% (v/v) acetonitrile in water containing 0.1% (v/v) TFA; flow-rate, 3 ml/min; temperature, 80°C; column inlet pressure, 14.7 MPa; sample size, 10 ng; detection at 210 nm. From Ref. [34].

efficiency in a major way. Yet, there are numerous other considerations, such as environmentally correct low solvent consumption, high mass sensitivity, low heat capacity, and easy conjugation with other analytical techniques, e.g. capillary electrophoresis and mass spectrometry, that may contribute to the progress in the miniaturization of HPLC.

4. Operational parameters

4.1. Temperature

Temperature has been an underrated operational parameter in HPLC and the potential advantages of elevated column temperatures, such as enhanced kinetic and transport properties, have yet to be widely exploited for rapid analysis of biological macromolecules by HPLC. The disinterest in controlling temperature in HPLC is due to the relatively small effect of temperature on selectivity and retention in HPLC of small molecules and to the fact that the magnitude of retention in liquid chromatography, unlike in gas chromatography, can conveniently be modulated by manipulating the mobile phase composition. Thus the relatively high complexity of the instrumentation required

for appropriate temperature control in HPLC at high temperatures and the lack of columns that are stable enough to withstand the harsh conditions at elevated temperatures over an extended period of time have deterred the development of suitable equipment.

Peptides and proteins are delicate molecules which can degrade at elevated temperatures. Therefore they are usually chromatographed at ambient or even sub-ambient temperatures in order to preserve the integrity of the molecular structure. Only recently has the need for rapid analysis of biological macromolecules drawn attention to the use of elevated column temperatures, so that analytical HPLC is routinely carried out at temperatures 20–40°C above ambient temperature [51]. Results obtained by using columns packed with thermally and hydrolytically stable micropellicular stationary phases at temperatures up to 120°C [34,40,42] have demonstrated the advantages of high-temperature HPLC in rapid protein separations.

The effect of temperature on the separation of large molecules has been investigated both theoretically and experimentally [26,42]. At high temperatures the mobile phase viscosity is reduced as shown in Fig. 12, and concomitantly, the diffusivity of the eluite is enhanced. Moreover, the sorption kinetics of the eluite is also accelerated with increasing temperature. Consequently, column efficiency is expected to be higher at elevated column temperatures and this is seen in Fig. 13 from the Van Deemter plots of data obtained at high flow velocities. The effect of temperature on the separation of standard proteins and of the peptides present in the tryptic digest of β-lactoglobulin B obtained on HY-TACH columns is shown by the chromatograms in Figs. 14 and 15, respectively. The two sets of chromatograms illustrate that at super-ambient temperatures where high flow velocities are permitted the separation time was significantly reduced without compromising the resolution as discussed in the Theory section.

In addition to the above described means of reducing particle size and selecting a stationary phase configuration of relatively low mass transfer resistance (pellicular or gigaporous sorbents), the use of elevated column temperature may serve as another useful concomitant for speeding up a separation. Of course, chromatography at high temperatures is a catholic approach to rapid analyses and can be carried out by using any column packed with a sufficiently stable stationary phase.

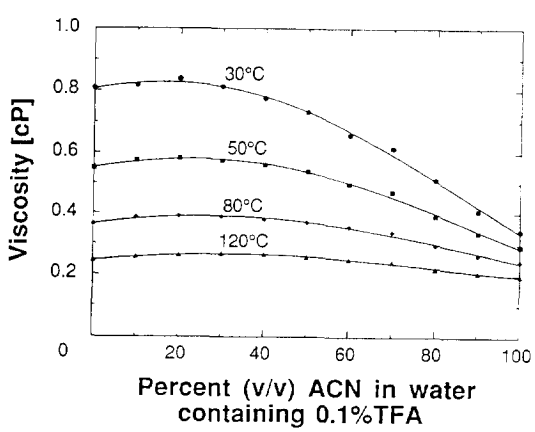

Fig. 12. Viscosity of acetonitrile–water mixtures at different temperatures as a function of the composition. A 30 × 4.6 mm column packed with 2-μm pellicular ODS-silica was used for the measurements and the mixtures contained 0.1% (v/v) TFA. From Ref. [42].

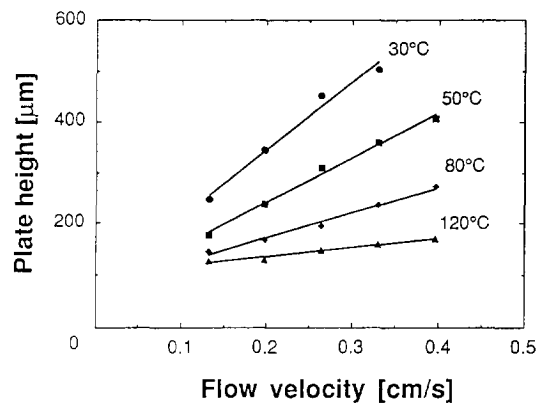

Fig. 13. Plots of plate height versus linear flow velocity measured with ribonuclease A at elevated temperatures in the domain of high reduced flow velocities. Column, 30 × 4.6 mm, packed with 5-μm macroreticular cross-linked polystyrene. Concentration (v/v) of acetonitrile in water containing 0.1% (v/v) TFA: ● = 25%; ■ = 23.5%; ◆ = 22%; ▲ = 18%. From Ref. [42].

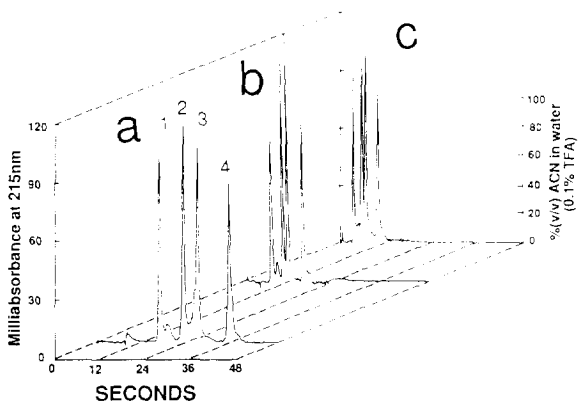

Fig. 14. Chromatograms of standard proteins obtained on a 30 × 4.6 mm I.D. column packed with 2-μm pellicular ODS-silica under three different temperature and flow-rate conditions: (a) 30°C, 2 ml/min; (b) 80°C, 4 ml/min; (c) 120°C, 5 ml/min. Linear gradient of 2.5 ml from 10 to 90% (v/v) acetonitrile in water containing 0.1% (v/v) TFA. Peaks: 1 = ribonuclease A; 2 = cytochrome *c*; 3 = lysozyme; 4 = β-lactoglobulin B. From Ref. [42].

Column stability at the anticipated operating temperatures over an extended period of time is a prerequisite of high-temperature HPLC. Columns packed with pellicular stationary phases have been shown not to change their properties at temperatures up to 120°C for at least 1000 h of operation [42]. Though the thermal stability of

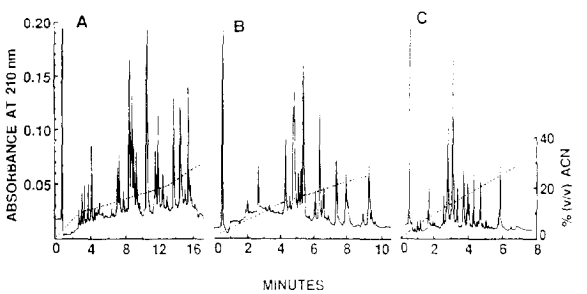

Fig. 15. Effect of temperature and flow-rate on the separation of the tryptic fragments of β-lactoglobulin B. Column, 75 × 4.6 mm, packed with micropellicular C_{18} silica; temperature and flow-rate, (A) 25°C, 0.8 ml/min; (B) 50°C, 1.3 ml/min; (C) 80°C, 2.0 ml/min. The initial column inlet pressure was 34.3 MPa in each case. Sample: 4 μg of reduced and S-carboxymethylated β-lactoglobulin B digest in 20 μl. From Ref. [39].

porous stationary phases especially silica-based porous sorbents in contact with the mobile phase is generally not satisfactory, porous siliceous sterically protected bonded C_{18} stationary phases have been reported to withstand the operating conditions at elevated temperatures up to 90°C [30].

So far the HPLC analysis of proteins at elevated temperatures has been carried out almost exclusively by reversed-phase chromatography. Since this technique employs denaturing conditions even at ambient temperature, further denaturation due to the combined use of the acidic hydro–organic medium, strongly hydrophobic stationary phase, and high temperatures does not cause further complications. Protein analysis at elevated temperatures by using other branches of HPLC may be associated with undesirable conformational changes or further chemical reactions lest the separation is sufficiently fast to avoid such interferences.

HPLC at temperatures above the atmospheric boiling point of the mobile phase may be termed "superheated" liquid chromatography [26] that in many respects, is similar to supercritical fluid chromatography. Both take advantage of enhanced transport properties of the mobile phase to obtain high chromatographic speed and efficiency [26]. The advantages of using superheated or supercritical fluid as the mobile phase over the traditional eluents in HPLC at ambient temperature illustrated in terms of column efficiency by the Van Deemter plots in Fig. 16. Upon the availability of appropriate instruments and columns, superheated liquid chromatography may be superior to supercritical fluid chromatography by offering a greater choice of mobile phases and thus facilitating the modulation of chromatographic retention and selectivity in a wide range [26]. Supercritical fluid chromatography, on the other hand, may be more suitable for the analysis of thermolabile substances and in those instances when the peculiar solvent properties of supercritical carbon dioxide can be utilized for enhanced chromatographic efficiency.

At elevated temperatures undesirable on-column reactions may be accelerated to an extent

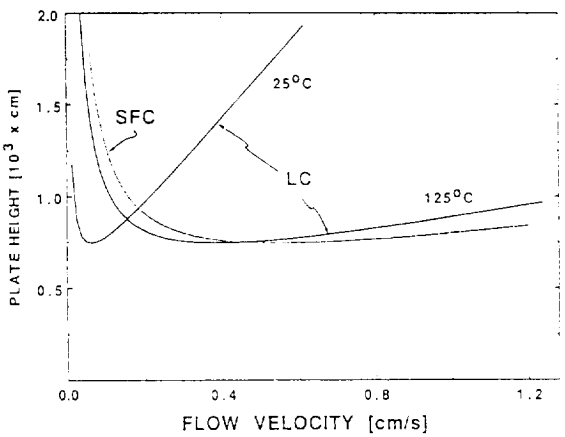

Fig. 16. Van Deemter plots for conditions typical in supercritical fluid chromatography, superheated liquid chromatography, and liquid chromatogaphy at ambient temperatures. Porous 3-μm particles. The diffusivities of the eluite in the three case are 10^{-4}, 10^{-4} and $5 \cdot 10^{-6}$ cm^2/s, respectively. From Ref. [26].

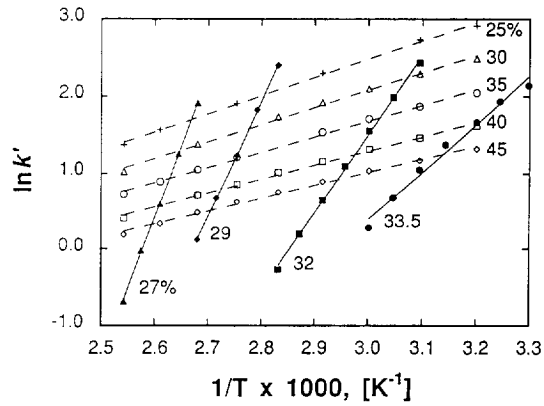

Fig. 17. Plots of the logarithmic retention factor versus the reciprocal absolute temperature in the reversed-phase chromatography of lysozyme (solid lines) and nitrobenzene (dashed lines). The percentage of acetonitrile in the aqueous mobile phase is shown for each set of data. Column, 30 × 4.6 mm, packed with 5-μm macroreticular cross-linked polystyrene; flow-rate: 1 ml/min. In the chromatography of lysozyme the mobile phase contained 0.1% (v/v) trifluoroacetic acid. From Ref. [42].

that may interfere with the chromatographic process. The interplay of the retention process and the on-column reaction is quantitatively measured by the Damköhler number (*Da*), which is defined as the ratio of the residence time in the column to the relaxation time for the on-column reaction [52]. Large *Da* values indicates that the reaction is completed in the column so that only the product(s) of the reaction exit at the column outlet. On the other hand, when *Da* is much smaller than unity, the reaction, such as protein degradation, does not take place to any significant extent during the residence time of the reactive eluite. Although the rate of most on-column reaction increases, the retention time decreases with increasing temperature. Consequently, under conditions of rapid analysis *Da* may remain small and thus significant degradation of the sample may not take place in the column [26].

The retention enthalpies of large molecules are generally greater than those of small ones. This is seen in Fig. 17 from the Van 't Hoff plots of lysozyme and nitrobenzene data obtained in reversed-phase chromatography. For large molecules, therefore, their retention is generally quite sensitive to the changes in temperature that can thus be an effective retention modulator besides affecting the efficiency of the column. This suggests that temperature programming could be a powerful adjunct or even an alternative to mobile phase gradients in the analytical separation of macromolecules by reversed-phase chromatography with columns having low heat capacity.

Temperature programming is eminently suitable for eluting sample components having a wide volatility range and has been widely used in gas chromatography [53]. However, this anisocratic elution mode [21] is rarely used in liquid chromatography although for the separation of large molecules having high retention enthalpies, temperature programming is offers certain advantage. It has been observed, for instance, that the change in selectivity upon changing the temperature is different from that upon changing the mobile phase composition in the separation of peptides [54]. Consequently temperature programming could complement gradient elution. Further, temperature programming might open a narrow elution window for closely related species

and permit a finer tuning of their retention behavior than gradient elution.

Nevertheless, temperature programming can find wide practical applications only when appropriate narrow- or micro-bore columns having low heat capacity and high thermal conductivity are available to facilitate rapid heating and cooling without untoward radial temperature profiles. If packed fused-silica capillaries prevail in the future of HPLC, they may elicit a growing use of temperature programming since it is much easier to program the column temperature than to generate eluent gradient at flow-rates of a few μl/min or nl/min [55].

4.2. Gradient time and flow velocity

As mentioned above, gradient elution is the widely used anisocratic elution mode in the HPLC of peptides and proteins. The magnitude of retention under gradient conditions depends on the flow velocity, the gradient time and volume as well as the starting and final mobile phase compositions. In gradient elution with linear solvent strength gradient, the retention factor at the column midpoint, $\bar{k}$, is given by [28]

$$\bar{k} = \frac{ut_G}{1.15L \log \frac{k_0}{k_f}} \quad (13)$$

Eq. 13 indicates that the magnitude of retention can be easily modulated by changing the gradient time and flow velocity.

With gradient elution, the total analysis time is the sum of the retention time of the last peak and the column regeneration time, i.e., the time it takes to return the column to its initial condition after the gradient run is completed. Columns packed with micropellicular or gigaporous stationary phases require much shorter reequilibration times than those packed with traditional porous stationary phases [43,56]. Thus, such stationary phases of advanced configuration are particularly suitable for use with rapid gradient runs as both the actual separation time and the regeneration time can be significantly reduced.

Whereas gradient elution is used in the separation of sample components having retention factors widely different, in the chromatography of very closely related proteins, isocratic elution with highly efficient columns may be more appropriate as shown in Fig. 11. In addition to the greater resolution, the use of isocratic conditions for closely related sample components has the advantage that the column need not be regenerated and concomitantly the analysis time may be reduced.

5. Instrumentation

Rapid HPLC requires not only stationary phases of low mass transfer and kinetic resistances, but also specially designed instrumentation capable to generate fast eluent gradients and provide temperature control over a wide super-ambient temperature range [8]. Other demanding features of the instrument having low overall system dead volume include a precise sample introduction device with low dispersion as well as a highly sensitive detector with short response time and small flow-cell volume. For separations with high speed and high efficiency by using narrow-bore or micro-bore columns, the demand for very small extra-column dispersion is extremely stringent and consequently the extra-column volume has to be drastically reduced.

As mentioned before it is necessary to control the column temperature in liquid chromatography in order to exploit the potential of high-temperature HPLC and to obtain reproducible results. The HPLC unit must have means for heating the column to the desired temperature without radial temperature gradients that are known to deteriorate its efficiency [57–59]. In Fig. 18 two alternative configurations are shown of an HPLC unit designed for use at elevated temperatures. In configuration A the entire fluid line between pumps and column is located inside the oven, on the other hand, in configuration B the heat exchanger, the mixer, and the injector are outside the oven and the eluent is heated immediately after leaving the injector to the oven temperature by a flash-heater [42]. Selec-

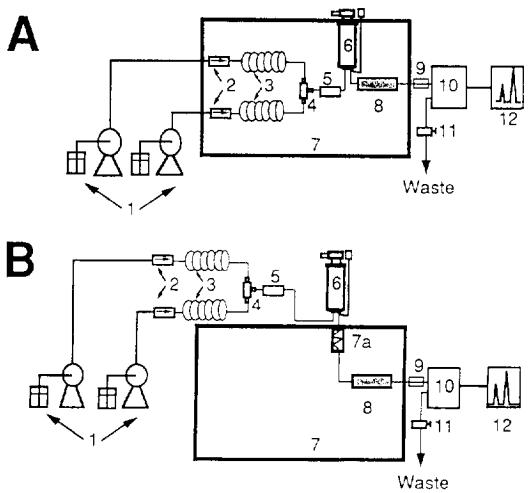

Fig. 18. Flow sheets of the two configurations of the liquid chromatograph for use at temperatures over 100°C with the sample injector inside (A) or outside (B) the oven. 1 = Reservoirs and pumps; 2 = in-line filters; 3 = heat exchangers; 4 = mixer; 5 = inverse in-line filters; 6 = sampling valve; 7a = flash heater; 7 = high-temperature oven; 8 = column; 9 = cooling device; 10 = UV detector; 11 = restrictor; 12 = data processor. From Ref. [42].

tion of the HPLC unit configuration depends on the preferred operating conditions. Configuration A is suitable only for the analysis of samples dissolved in solvents with boiling point higher than the column temperature. In contrast, configuration B places no restriction on the sample solvent and provides the flexibility and convenience needed for quantitative sample introduction over a wide range of super-ambient column temperatures. However, in this case the extra-column dead volume is relatively large due to the flash heater placed between the injector and column.

For high-temperature HPLC the instrumental specifications call for a heat exchanger that can bring the eluent rapidly to column temperature. Specially made thin-walled capillary tubing, called "serpentine tubing", is often employed in such applications [60]. The design of the HPLC system is particularly critical when temperature programming is used, because it requires instantaneous temperature change of both the column and the incoming mobile phase. In general, inadequate heat exchange due to poor construction of the instrument can annihilate the advantages of HPLC at high temperatures [58].

Steep eluent gradients and short columns are often used in the rapid separation of peptides and proteins. The generation of such gradients in a reproducible fashion requires precise and pulseless eluent delivery from the pumps, an efficacious mixing device and low dwell volume. It is recalled that the dwell volume is the volume of the connecting tubing between the inlets of the mixer and the column. Large dwell volume not only delays and distorts the gradient, but also increases the time required for reequilibration of the column. Systems having a dwell volume low enough to obtain dwell times as short as 10 s or less have been used in rapid analysis of peptides and proteins [6,7,56].

One of the most important potential applications of rapid HPLC is in on-line process monitoring, where the design of the sampling system is of particular importance. Especially in-process analysis in biotechnology can benefit from rapid HPLC that can be considered as the ultimate "multi-analyte sensor". In biotechnological applications on-line monitoring of the concentration of certain substances in the fermentation broth usually requires a more elaborate sampling system than that in the effluent from some chromatographic or membrane purification steps. "Dirty" samples from the fermentor, for instance, may require the removal of particulate matter by filtration or dialysis [61,62]. Sometimes it is necessary to dilute the sample before introducing it into the column. Generally, the sampling device should be sterilized in order to avoid contamination of the process stream or the fermentor.

In rapid HPLC separations the band width may be on the time scale of a few seconds and the time constant of the detection system should be less than 100 ms. In the future, separations may be carried out on the millisecond scale and then the time constant of the HPLC unit has be proportionally smaller. In any case, in such applications computers are indispensable for data acquisition and processing. With the aid of high-speed computers, data processing is no

longer the speed-limiting step in the comprehensive analysis scheme. The computer also plays a very important role in the automation of HPLC systems by facilitating the remote control of the instrument and of data collection. Thus the reproducibility and accuracy of the results are enhanced and the analytical results are made available without delay. It should be emphasized again, however, that further progress in high-speed HPLC will inexorably call for concomitant advances in prechromatographic sample treatment.

Acknowledgements

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